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CHROMOSOMAL VARIATION IN POPULATIONS OF *DROSOPHILA PSEUDOOBSCURA* WHICH INHABIT NORTHERN MEXICO

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FIFTEEN gene arrangements in the third chromosomes are known in natural populations of *Drosophila pseudoobscura*. Each arrangement is geographically limited to a part of the distribution area of the species. Some of the arrangements are narrowly endemic and others more or less widespread. The population of any one locality may be described in terms of the relative frequencies in it of various gene arrangements. By and large, the composition of populations which inhabit geographically close localities is more similar than that of populations which live far apart. Comparison of a series of populations usually reveals geographic gradients (clines) in the frequencies of gene arrangements; some arrangements become more and others less frequent as one moves in a given direction through the distribution area of the species. An examination of the gradients discloses, however, a very significant fact, namely, that the slope of a gradient may be steeper in some regions than in others. For example, populations that live on the Pacific Coast of the United States, west of the Sierra Nevada-Cascades mountain chain, show high frequencies of the Standard gene arrangement, but this arrangement rapidly becomes infrequent as one moves eastward from these mountains into the Great Basin.

The non-uniformity of the gradients makes it possible to delimit several races, or subspecies, within the species *Drosophila pseudoobscura*. The previously published data (Dobzhansky, 1944) have indicated four such races. A race in which chromosomes with the Standard gene arrangement are frequent, inhabits the Pacific Coast, from British Columbia to Lower California. Arrowhead gene arrangement is prevalent in populations of the Great Basin and Colorado Plateau. Populations of the Rocky Mountains in Colorado and of Texas show high frequencies of Pikes Peak chromosomes. The information available on the populations of Mexico and Guatemala is scanty, but it suggests that the race which occurs from central Mexico to Guatemala is the most distinctive of all. Standard and Arrowhead are unknown, and Pikes Peak is rare. In this race chromosomes with the Cuernavaca arrangement, which does not occur in the United States, are frequent, and so are Santa Cruz, Tree Line, and Estes Park arrangements which are relatively rare north of the Rio Grande.

Professor Herman T. Spieth[1] has very kindly collected and sent to the writer samples of populations of *Drosophila pseudoobscura* from the states of Chihuahua, Durango, and Zacatecas, in northern Mexico. The region from which these samples came is very nearly a terra incognita as far as the knowledge of races of *Drosophila pseudoobscura* is concerned. Despite the smallness of most of these samples, their examination sheds light not only on the composition of the populations of northern Mexico, but also on the race structure of the species as a whole. The writer is greatly indebted to Professor Spieth for collecting the material, and to Mr. B. Spassky and Mr. Morris Foster who made the necessary crosses and prepared the smears of the salivary glands in which the chromosomes were examined.

[1] While with the D. Rockefeller expedition of the American Museum of Natural History.

The Populations of Chihuahua, Durango, and Zacatecas

The numbers of chromosomes with different gene arrangements found in the population samples collected by Professor Spieth are reported in Table 1. The samples from Santa Clara Mountains and from Chihuahua City, in the state of Chihuahua, are also included in the table for completeness. These samples were obtained through the courtesy of Professor J. T. Patterson, and their composition was published by Dobzhansky (1944).

TABLE 1

Numbers of Chromosomes With Different Gene Arrangements Found in the Samples from the States of Chihuahua, Durango, and Zacatecas

Locality	Arrowhead	Pikes Peak	Chiricahua	Santa Cruz	Tree Line	Olympic	Santa Barbara	n
Santa Clara Mts., Ch.	3	6	30	..	..	1	..	40
Chihuahua City, Ch.	18	72	142	7	3	2	..	246
Parral, Ch.	..	2	2	..	..	..	..	4
Santa Barbara, Ch.	6	40	229	11	3	1	10	300
Encino, Du.	..	..	4	..	..	..	..	4
El Toscate, Du.	..	1	7	..	..	..	..	8
Palos Colorados, Du.	..	4	43	12	3	..	..	62
Nombre de Dios, Du.	..	2	1	1	..	..	..	4
Otinapa, Du.	..	..	4	..	..	..	..	4
Coyote, Du.	..	2	12	..	..	..	..	14
Guadalupe, Zac.	..	2	3	3	3	1	..	12

Seven different gene arrangements in the third chromosome occur in the populations of the region under consideration. Six of them have been known previously (Dobzhansky, 1944). The seventh, found in the population of Santa Barbara and accordingly to be referred to by that name, has never been encountered. The Santa Barbara arrangement differs from the well known and widely distributed Chiricahua arrangement by a single small inversion which includes the part of the chromosome between sections 66D and 67E of the standard map (see Plate 1, Dobzhansky and Sturtevant, 1938). It can be concluded that Santa Barbara arose from Chiricahua

by a single inversion step, and must accordingly be placed in the phylogenetic tree of the gene arrangements (Dobzhansky, 1944, fig. 5) next to Chiricahua and San Jacinto arrangements. Like San Jacinto, which is an endemic chromosomal type known from a single locality, Santa Barbara turned up in a single sample only. However, since ten chromosomes of this type have been found in that sample (Table 1), the Santa Barbara arrangement

TABLE 2

FREQUENCIES (IN PER CENT) OF CHROMOSOMES WITH DIFFERENT GENE ARRANGEMENTS IN POPULATIONS OF SOUTHWESTERN UNITED STATES AND MEXICO

Region	Standard	Arrowhead	Pikes Peak	Chiricahua	Santa Cruz	Tree Line	Olympic	Estes Park	Cuernavaca	Santa Barbara	n
San Jacinto, Calif.	41.5	25.6	...	29.2	0.3	3.4	...	...	...	...	11647
Majave and Colorado Deserts, Calif.	15.4	73.2	...	10.0	0.3	1.0	...	...	...	...	370
So. Arizona	1.9	79.9	4.1	13.7	...	0.3	...	...	...	...	314
So. New Mexico	0.2	77.5	17.1	4.2	...	1.0	...	...	...	...	404
Trans-Pecos, Texas	0.9	27.4	65.6	2.4	...	3.3	...	0.5	...	...	212
No.-central Texas	...	21.5	70.2	...	...	6.1	1.4	0.7	...	...	1315
So.-central Texas	0.2	11.7	70.3	...	...	12.4	5.3	0.7	...	...	418
Valley region, Texas ..	...	3.3	76.7	...	...	16.7	3.3	...	...	...	30
Central Chihuahua	...	7.3	27.3	60.8	2.4	1.0	1.0	...	...	...	286
So. Chihuahua–No. Durango	...	1.9	13.6	76.6	3.5	0.9	0.3	...	...	3.2	316
So. Durango	...	...	9.5	71.4	15.5	3.6	...	...	...	...	84
Zacatecas	...	...	16.7	25.0	25.0	25.0	8.3	...	...	...	12
Nuevo Leon	...	2.9	8.8	...	2.9	35.3	47.1	2.9	...	...	34
Michoacan	...	...	3.7	...	40.7	18.5	3.7	...	31.5	...	54
Central Mexico	...	...	...	1.3	0.4	24.3	6.1	14.3	50.9	...	230

is an integral part of the population of at least one locality, and not a form just arisen by mutation. Santa Barbara is interesting in two further respects. Namely, the inversion by which it arose from Chiricahua is the shortest among the twenty-one inversions in the third chromosome known in natural populations of *Drosophila pseudoobscura*, and the breakage points giving rise to Santa Barbara lie in the part of the chromosome in which no other "natural" breaks were known, a fact which sug-

gested that the break distribution along the length of the chromosome is not at random (Novitski, 1946).

The samples from Chihuahua and Durango agree in showing a predominance of the Chiricahua gene arrangement, with Pikes Peak, Santa Cruz, and Arrowhead being also fairly common. Nowhere else in the known distribution area of the species have populations of this composition been found. Northern Mexico is, consequently, inhabited by an endemic race of *Drosophila pseudoobscura*. The characteristics of this race, and the extent of the territory occupied by it, stand out most clearly if one compares the frequencies of the gene arrangements in northern Mexico with those in adjacent regions. Such a comparison is presented in Table 2. In this table, the samples from Santa Clara mountains and from Chihuahua City (*cf.* Table 1) are grouped together under "Central Chihuahua," those from Parral, Santa Barbara, Encino, and El Toscate under "Southern Chihuahua and Northern Durango," and those from Palos Colorados, Nombre de Dios, Otinapa, and Coyote under "Southern Durango." The data for the other regions are taken, with modifications, from Dobzhansky (1944).

Delimitation of the Races of *Drosophila pseudoobscura*

Examination of Table 2 and Figure 1 discloses both the existence of geographic gradients in the frequencies of some gene arrangements, and of more or less pronounced breaks in these gradients in certain regions. These breaks permit delimitation of the races.

As stated above, high frequencies of the Chiricahua arrangement are characteristic of the populations of Chihuahua and Durango (60–75 per cent). Northward from there, in southern Arizona and southern New Mexico, these frequencies rapidly fall below the 10 per cent level; only in the samples from Huachuca Mountains and from Sonoita, in Arizona just north of the Mexican border, have frequencies of about 30 per cent Chiricahua

been found (*cf.* Dobzhansky, 1944, p. 86). In northern Arizona and northern New Mexico, Chiricahua chromosomes are rare. In Texas, Chiricahua does not occur, except rarely in the Trans-Pecos area, which is the west-

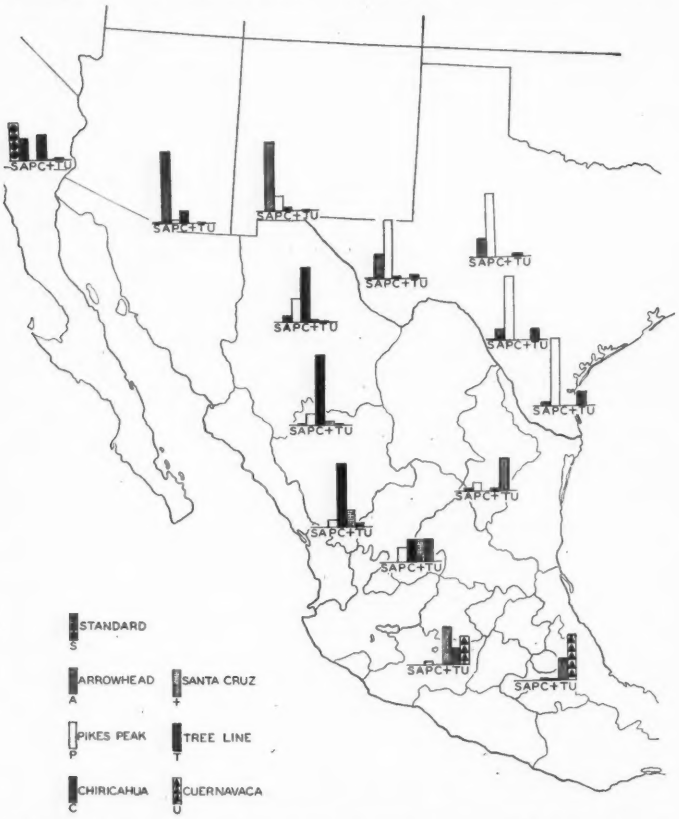

FIG. 1. Relative frequencies of certain gene arrangements in populations of *Drosophila pseudoobscura* in Mexico and the southwestern United States. The heights of the columns on the map are proportional to the frequencies of the respective gene arrangements in the various regions.

ernmost part of the state. The situation in the territories which lie to the West and Northwest from Chihuahua is unclear, because the Mexican state of Sonora remains unexplored. It is, however, known that Chiricahua chro-

mosomes occur, with frequencies varying between 10 and 30 per cent, on the Pacific Coast from British Columbia to Lower California.

In central Mexico (including Michoacan) and in Guatemala, Chiricahua chromosomes are rare or absent. The boundary between the Central-Mexican and the North-Mexican (Chihuahua and Durango) races lies probably somewhere south of the state of Zacatecas, since a small sample of chromosomes from the latter state contains about 25 per cent of Chiricahua, and no Cuernavaca chromosomes. High frequencies of Cuernavaca are characteristic of central Mexico.

The situation in northeastern Mexico is still puzzling, owing to scarcity of information. A sample of thirty-four chromosomes from Nuevo Leon (San Josecito) differed sharply from anything else known in the species. This sample contained no Chiricahua chromosomes, which set it apart from populations of Chihuahua and Durango, and no Cuernavaca chromosomes, which distinguished it from populations of central Mexico.* On the other hand, the Nuevo Leon sample contained 45 per cent of Olympic chromosomes, which is a much higher frequency of this chromosomal type than found anywhere else. This suggests the existence of a very distinctive race of *Drosophila pseudoobscura* in northeastern Mexico.

Apart from the high frequency of Chiricahua chromosomes, the North-Mexican race is characterized by the rarity or absence of certain gene arrangements which are frequent in other races. The absence in the samples from Chihuahua, Durango, and Zacatecas of Cuernavaca chromosomes has already been pointed out; the boundary between the North-Mexican and the Central-Mexican races must be rather sharp, although the precise location of this boundary is as yet unknown. The frequencies of Pikes Peak, which is a very common chromosomal type in Texas

* Nothing is known about the populations of Coahuila, a state which intervenes between Chihuahua and Nuevo Leon. The maps on pp. 91 and 93 in the paper of Dobzhansky, 1944, are in error showing the presence of four different chromosome types in Coahuila.

and in the southern Rocky mountains, dwindle rapidly south of the Mexican border, although some of these chromosomes occur as far south as Michoacan. The Arrowhead arrangement, which is prevalent in the Great Basin and on the Colorado Plateau, behaves like Pikes Peak, except that the downward gradient to the south is much steeper (about 7 per cent in central Chihuahua, none from southern Durango southward). Finally, the Standard gene arrangement, which is characteristic of the Pacific Coast race, has not been found in Mexico at all.

Conclusions and Summary

The species *Drosophila pseudoobscura* may be tentatively subdivided into the following races:

1. *Pacific Coast race.* The commonest gene arrangements are Standard, Arrowhead, and Chiricahua, usually in that order. Tree Line, Santa Cruz, Olympic, and Pikes Peak are usually present but more or less rare. This race extends from coastal British Columbia to at least the northern part of Lower California, and from the Ocean eastward up to and including the Cascades, Sierra Nevada, and Sierra Madre mountains. In the Coast Ranges south of the San Francisco Bay, and on the islands off the California Coast, the populations show higher frequencies of Santa Cruz chromosomes than is the case farther east and north, but these coastal populations are not sufficiently distinct from others to be regarded as a separate race.

2. *Great Basin race.* Arrowhead is the prevalent chromosomal type. In some regions (northern Arizona, southern Utah, southwestern Colorado, and northwestern New Mexico) the populations contain few or no chromosomes other than Arrowhead. Elsewhere in the distribution area of this race, Chiricahua, Standard, and Pikes Peak occur as admixtures to Arrowhead. This race lives to the east of the Sierra Nevada-Cascades mountain chain, from eastern British Columbia to Arizona and western New Mexico. Its eastern boundary is not well known; it probably extends to the northern Rocky Mountains, while

the southern Rockies (in New Mexico) appear to be inhabited by populations transitional between the Great Basin and the Great Plains races.

3. *Great Plains race.* Pikes Peak is the commonest gene arrangement. Arrowhead is fairly common, and Tree Line, Estes Park, and Olympic are present but are nowhere very frequent. This race occupies the eastern portion of the distribution area of the species in the United States. It is best developed in Texas and in the Front Range of the Rocky Mountains in Colorado.

The three races discussed so far have in common the presence of the Arrowhead gene arrangement in fairly high concentrations. In the following four races Arrowhead is rare or absent.

4. *North-Mexican race.* The commonest gene arrangement is Chiricahua. Pikes Peak, Santa Cruz, Tree Line, Arrowhead, and Olympic are admixtures which diffuse into this race from its neighbors. The North-Mexican race is known to occur in the states of Chihuahua, Durango, and probably Zacatecas. Although the exact limits of the distribution of this race are not known, it appears to be rather sharply segregated from the Great Basin race in the north and from the Central-Mexican race in the southeast.

5. *Central-Mexican race.* Cuernavaca, Tree Line, and Estes Park are the principal constituents of the populations, with Olympic, Santa Cruz, Oaxaca, and Chiricahua as admixtures. It occurs in the Mexican states of Mexico, Morelos, Puebla, Vera Cruz, and Oaxaca.

The following two races are very tentative, since their characterization is based on study of small samples.

6. *Northeast-Mexican race.* A sample from the state of Nuevo Leon indicates the existence of a race in which Olympic and Tree Line are the prevalent gene arrangements.

7. *Michoacan-Guatemala race.* Santa Cruz and Tree Line are the commonest gene arrangements, with Cuernavaca being fairly common. Populations of this com-

position are known from the northeastern part of the state of Michoacan in Mexico and from western Guatemala. Until the intervening territory is studied, it can not be decided whether this race has a continuous or a discontinuous distribution. The sample from the state of Zacatecas (Table 2) appears to be transitional between the Michoacan-Guatemala and the North-Mexican races but closer to the latter.

The above tentative classification of the races of *Drosophila pseudoobscura* is based on a single trait—the gene arrangement in the third chromosome. Although this trait is biologically important (Dobzhansky, 1947), it is possible that other variable traits might suggest other subdivisions of the species, just as the classifications of human races based on skin color, cephalic index, blood groups, and other traits frequently do not agree with each other.

The distribution regions of the races of *Drosophila pseudoobscura* in Mexico agree tolerably well with the biotic provinces of that country as outlined by Goldman and Moore (1945). Namely, the North-Mexican race seems to correspond to the Sierra Madre Occidental and Chihuahua-Zacatecas provines, the Central-Mexican race to the Transverse Volcanic province, and the Northeast-Mexican race to the Sierra Madre Oriental and perhaps the Tamaulipas provinces. The Michoacan-Guatemala race is the only one which seems to have no equivalent in the Goldman and Moore classification; however, more data is needed to elucidate the nature and distribution of this race.

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DENSITY OF FEATHER PIGMENT IN RHODE ISLAND REDS[1]

PROFESSOR F. A. HAYS, CAROL H. WHITE AND RUBY SANBORN

Feather color in fowls has been shown to depend on the character and amount of pigment and upon the size, shape and arrangement of pigment granules. Attempts to breed Rhode Island Reds for plumage color have demonstrated a wide color range which offers great difficulties in efforts to make phenotypic classification. Measuring pigment density colorimetrically may have some value in classifying birds for plumage color and thus assist in a study of the genetics of plumage color.

Review of Literature

Bohren, Conrad and Warren (1943) have reported on an extensive study of feather pigments in domestic fowl and have reviewed the literature on the subject. It is only necessary therefore to make reference in this report to studies concerned with the particular phase being considered.

Lloyd-Jones (1915) suspended red pigeon feathers in 2.5 per cent. NaOH and after heating found that the solution had taken on the chestnut brown color of the feathers. He observed that practically all the pigment in the feathers was dissolved.

Ladebeck (1922) made use of both NaOH and KOH as solvents for pigments in chicken feathers. Concentrations of 2 per cent. and .2 per cent. were tested. He observed that yellow pigments dissolved readily and that red pigment dissolved when heat was applied. Black pigment was essentially insoluble in dilute alkalies.

Steele (1937) digested feathers in 2.5 per cent. solution of NaOH by boiling for 15 minutes. Concentration of feathers ranged from one gram of feathers to 1,000 cc

[1] Contribution No. 640 from the Massachusetts Agricultural Experiment Station.

to one gram of feathers to 8,000 cc. The more dilute solution gave more favorable results. An electrometric colorimeter was used to test the relative amounts of melain in samples of feathers.

Bohren, Conrad and Warren (1943) tested the solubility of pigments in feathers using hot and cold HCl of about 22 per cent. concentration and 2 per cent. NaOH. These workers observed that yellow pigment dissolved very readily in dilute alkali, red pigment dissolved on heating but black pigment was essentially insoluble. In this study .2 grams of feathers were digested for two hours in 100 cc of NaOH. Only pigment of buff breeds dissolved in strong HCl. It was suggested that red feathers may differ from buff feathers in possessing an acid insoluble brown pigment.

Character of Birds Used

This study included Rhode Island Reds of an exhibition strain that were bred through four generations in a study of the mode of inheritance of plumage color. One line was selected for early sexual maturity while the other was selected for late sexual maturity.

Technique Employed

All feathers were taken from the mid-back region in both males and females as they approached sexual maturity. The control sample of feathers came from a male owned by a prominent breeder of exhibition Rhode Island Reds.

Pigment was extracted from a single control feather. After weighing, the feather was placed in a tube containing 10 cc of .2 per cent. KOH and boiled for five minutes. Pigment from all other feathers was digested in an amount of KOH proportionate to the weight of the feather. After boiling the solution was increased to its original volume by adding distilled water. Each solution was then filtered and colorimeter readings made with a Bausch and Lomb colorimeter with the control set at 15 mm.

Various questions arose regarding experimental error. First, there was the machine error which was checked as follows: Feathers were taken from twenty-two birds. For each bird the pigment solution was placed in the right and left cups of the colorimeter. One plunger was set at 15 mm and the other regulated until the color matched. The mean reading was found to be 15.16 mm. The significance of the 21 differences from 15 was calculated by Fisher's modification of Student's method. The value of t (.69) indicated an insignificant difference in the readings.

To test the variability in pigment density between feathers twenty-six birds were used. Two feathers were extracted from each bird. One solution was checked against the other. The series of 26 differences gave a t value of 2.55 which was significant and indicates that there was an important difference in pigment density in feathers from the same region in the same bird.

In this study .2 per cent. KOH solution was employed, while Ladebeck (1922) had tested both .2 per cent. and 2 per cent. solution of KOH and NaOH. Steele recommended 2.5 per cent. NaOH. Steele made use of two concentrations of pigment, namely, 1 gram of feather to 1,000 cc and 1 gram of feather to 8,000 cc. We averaged about one gram of feather to 540 cc.

Three different concentrations of KOH were tested; 2 per cent., .4 per cent. and .2 per cent. In all cases the .2 per cent. solution was used for a control. Three similar feathers were selected from each of 25 birds. The mean pigment density of the control was placed at 100, the mean for the .4 per cent. solution was 109.6 and the mean for the 2 per cent. solution was 103.3. A comparison of extractions by .2 per cent. KOH and .4 per cent. KOH gave a highly significant difference in favor of the .4 per cent. solution. Comparison of the .2 per cent. solution with the 2 per cent. solution gave only a significant difference. Solutions using .4 per cent. KOH and 2 per cent. KOH had a significant difference in density. The

possibility exists therefore that a concentration somewhat greater than .2 per cent. KOH might have been more effective as a pigment solvent.

Experimental Results: Association Between Sex and Pigment Density

Pigment density was determined on 118 males and 207 females. The mean density for the males was 66.86 per cent. of the standard. The mean for the females was 71.06 per cent. of the standard. There appeared to be a slight tendency for females to exhibit a higher degree of pigment density than males, but the differences were statistically insignificant.

Effect of Age at Sexual Maturity on Pigment Density

Age at sexual maturity may be determined accurately in females by trapnesting. In this study, however, trapnest records are available only on those females that were retained for the first laying year and not on the total population.

A study was made on the individual basis grouping the birds as "early" if they laid their first egg at an age of 215 days or younger and as "late" if the first egg was laid at 216 days or older. The 28 early individuals had a mean pigment density of 63.8 per cent. while the 50 late individuals had a mean density of 70.9 per cent. These differences are statistically significant and they suggest that individuals that come to sexual maturity late are more likely to exhibit a more dense pigmentation. It seems probable that the onset of early sexual maturity is antagonistic to high density of brown pigment in Rhode Island Reds.

Males and females in the lines bred for early and late sexual maturity were grouped together in the last three generations. In the early line some of the birds were not phenotypically early and not all birds in the late line were phenotypically late. A total of 136 males and

females in the early line had a mean pigment density of 72.1 per cent. while 101 males and females in the late line had a mean density of 75.8 per cent. In the last generation the mean pigment density of the two lines were 78.7 and 98.3 per cent., respectively. All differences were statistically significant and further confirm that early maturing birds are less likely to be intensely pigmented.

Inheritance Studies

For the purpose of discovering if there was any evidence of phenotypes for pigment density, a frequency distribution of all males and females is illustrated graphically in Fig. 1.

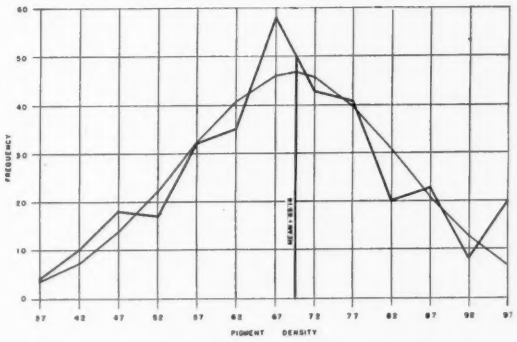

Fig. 1. Frequency distribution of four generations for pigment density by standard—N = 329.

By the X^2 test the population does not conform to a normal sample but seems to be made up of several phenotypes. A study of different generations led to evidence that there may be a division of pigment density types around the 75 per cent. class. As a result the offspring both male and female was tabulated from dams having a pigment density of 35 to 75 per cent. and the group having a density of from 76 to 99 per cent. These frequencies are recorded in Fig. 2. The figure shows that the mean pigment density in offspring was greater in the group from low density mothers. This fact suggests that

pigment suppressors must be in operation. The earlier observation that density of pigment is usually slightly greater in females than in males suggests either sex limitation or a sex-linked gene.

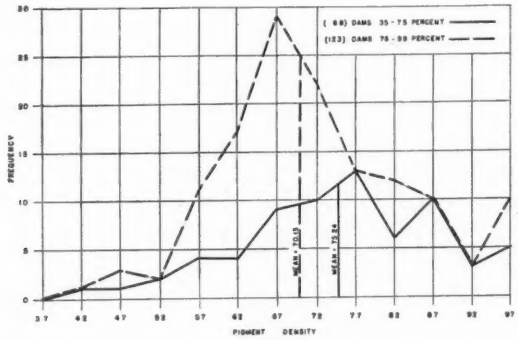

FIG. 2. Frequency distribution of sons and daughters from two possible types of dams.

REGRESSION OF OFFSPRING ON PARENTS FOR PIGMENT DENSITY

In this report records are available on 18 mothers that produced 139 daughters and on 13 mothers that gave 64 sons. All birds in the four generations were tested against the same standard for pigment density.

Fig. 3 shows that there was no regression of either sons or daughters on mothers for pigment density. The graphs also indicate that the daughters averaged to be slightly more densely pigmented than their brothers.

The total number of sires whose pigment density was tested was only five. It is not possible, therefore, from these limited data to measure the sire's contribution to the inheritance of his offspring. In fact, only two classes of sires were used. There were two with a pigment density of 55 to 59 per cent. and three with a pigment density of 75 to 79 per cent. These produced 56 daughters and 37 daughters, respectively. The frequency distribution of these two groups of daughters is given in

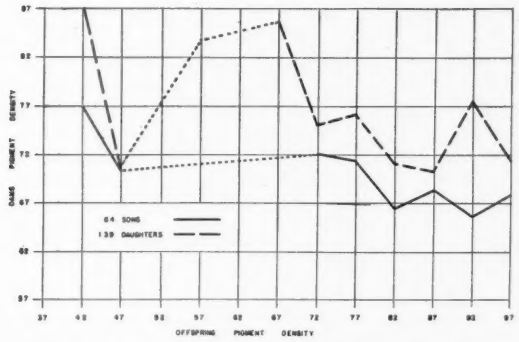

FIG. 3. Regression of sons and daughters on mothers.

Fig. 4. It will be observed that the low density sires gave daughters with a slightly higher mean density than was observed in the daughters of the higher density sires. The two frequency distributions shown in Fig. 4 were tested and found to belong to the same population. Some evidence is also afforded that females with a density up to about 75 per cent. may belong in a different population than those whose density is above 75 per cent.

Four tested sires gave 51 sons. The mean pigment density of the sons of the two low density sires was 74.58 per cent. Sons of the two high density sires averaged 66.72 per cent. Sons were slightly less densely pigmented than daughters and the same type of transmission was shown from sires to their sons and to their daughters.

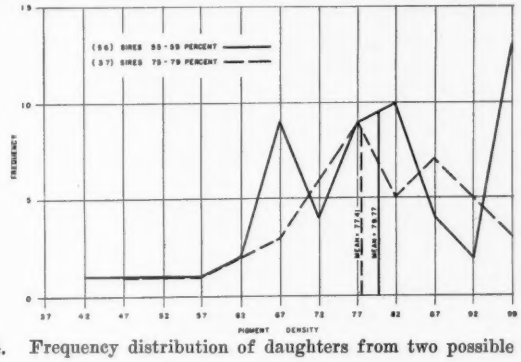

FIG. 4. Frequency distribution of daughters from two possible types of sires.

In all results reported so far pigment density was measured against feathers of an exhibition male. Density has been studied further using either the sire or the dam of a bird as the standard. By this method it is possible to discover how many birds in each family are more or less densely pigmented than the parent.

Pigment Density in Offspring Using Their Sire as Standard

In this study 91 offspring of two sires were checked for pigment density by using their sire as the standard. One sire had a pigment density of 79 per cent. against the original standard. The other sire had a density of 57 per cent. against the original standard. The first male had 47 offspring of which 32 were lower, 13 higher and 2 the same as he in density. The second male had 44 offspring of which 41 were lower and 3 higher than he in pigment density. These data further point to the dominance of light pigmentation over dense pigmentation.

Pigment Density in Offspring Using the Dam as a Standard

There was a total of 14 dams that were used as standards to measure the pigment density of their offspring. In a total of 145 offspring, 85 were less dense and 60 were more dense than their dam. These data suggest a closer association between mothers and offspring than was observed between sires and offspring in pigment density. This result would be anticipated if a sex-linked recessive gene is operating for high intensity.

Pigment Density within Families

Two families of high fecundity daughters were selected. One family consisted of 8 full sisters and the other of 10. Each of these females was tested against the original standard. They were unrelated families. The mean pigment density of the first group was 67.2 per cent.; that

of the second group was 40.8 per cent. In testing for significance of difference between the two means the *t* value was 5.68, which is highly significant. These data show that the members of a family tend to resemble each other more than they resemble the general population and thus suggest hereditary factors in operation.

Suggested Theory Regarding the Mode of Inheritance of Pigment Density

Two dominant pigment suppressors are suggested. These genes are named Dp, which is sex-linked, and Dp_1, which is autosomal. These genes appear to behave as multiple factors. According to this hypothesis there will be nine possible genotypes of males and six genotypes of females. The genotypes are the following:

Males	Females
Dp Dp Dp_1 Dp_1 very light	Dp Dp_1 Dp_1 very light
Dp Dp Dp_1 dp_1 light	Dp Dp_1 dp_1 medium
Dp dp Dp_1 Dp_1 light	dp Dp_1 Dp_1 medium
Dp dp Dp_1 dp_1 medium	Dp dp_1 dp_1 dark
Dp Dp dp_1 dp_1 medium	dp Dp_1 dp_1 dark
dp dp Dp_1 Dp_1 medium	dp dp_1 dp_1 very dark
Dp dp dp_1 dp_1 dark	
dp dp Dp_1 dp_1 dark	
dp dp dp_1 dp_1 very dark	

The following are some of the matings that have been studied to secure data on the mode of inheritance of pigment density. In all cases each bird was tested for pigment density against the original standard. A medium density male A156 was mated to four medium density females. Results are tabulated below. A lightly pigmented male W367 was mated to two medium hens. Daughters and sons were first compared in density with their dams. This is followed by a comparison with their sire. Expected and actual ratios are recorded for each mating.

In Table 1 the sons of male X156 offer the only discrepancy from expectation. It is questionable if the small number of sons, eighteen in all, furnishes adequate information. On the whole, there is very close conformity between observed and expected phenotypes.

TABLE 1

Sire X156 (Dp dp Dp_1 dp)
Dams X801, X1963, X2044 and X1479 (All Dp Dp_1 dp_1)
Compared with dams, sire constant

Expect	Daughters Observed X801	X1963	X2044	X1479	Totals
4 More than dam ..	4	4	1	6	15
3 Same as dam ...	3	2	4	3	12
1 Less than dam ..	1	0	1	0	2

$X^2 = .86$

Expect	Sons Observed X810	X1963	X2044	X1479	Totals
1 More than dam ..	0	No	1	1	2
3 Same as dam	1	Sons	5	5	11
4 Less than dam ..	1		4	0	5

$X^2 = 4.48$

Daughters of Male W367 (Dp dp dp_1 dp_1)
Dam W839 (Dp Dp_1 dp_1)

Expect	Observed
3 More than dam ..	3
1 Same as dam ...	1
0 Less than dam ..	0

$X^2 = 0$

Sire W367
Dam W683 (dp Dp_1 Dp_1)

Expect	Daughters Observed
0 More than dam ..	0
$2\frac{1}{2}$ Same as dam ..	3
$2\frac{1}{2}$ Less than dam .	2

$X^2 = .2$

Daughters Compared with Sire
Sire X156

Dams	Expect More	Same	Less	Observed More	Same	Less
X801	4	3	1	3	4	1
X1963	4	3	1	4	1	0
X1479	4	3	1	4	4	1
X2044	4	3	1	2	1	3
Totals	$14\frac{1}{2}$	11	$3\frac{1}{2}$	13	10	5

$X^2 = .889$

Sire W367

Dam	Expect More	Same	Less	Observed More	Same	Less
W839	1	2	1	2	1	1
			$X^2 = 1.5$			
W683	$2\frac{1}{2}$	$2\frac{1}{2}$	0	3	1	1
			$X^2 = 2.0$			

General Discussion

The data reported in this study furnish considerable information on the question of pigment density in Rhode Island Reds. There appears to be a sex difference in density in that females generally exhibit a slightly higher density than males. Although considerable error arises from using a single feather in density determinations, the number of birds studied in the four generations should overcome this error to a considerable extent.

Matings of more densely pigmented females gave daughters with a lower mean density than did matings of a group of females that were less dense themselves. This suggests that high density may be controlled largely by recessive genes and that very likely one sex-linked gene is in operation. The failure of many densely pigmented birds to breed true is also explained as is the appearance of densely pigmented offspring from some matings of sparsely pigmented parents.

In general, our data suggest that the Rhode Island Red color pattern in exhibition stock is built up of cumulative recessive genes for high density and that production bred stocks lack most of these high density genes.

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THE MALIGNANT CELL IN AN ENVIRONMENT OF NORMAL CELLULAR CONSTITUENTS—SELECTIVE SPECIFICITY IN A REPLACEMENT APPROACH TO CANCER AND RELATED PROBLEMS

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INTRODUCTION

IT has been desired to investigate the possibilities of modifying malignant cells by exposing them to normal cellular elements, and, through the processes of selective specificity and cellular dynamics, allowing the malignant cell to replace any normal constituents which may have been modified or depleted by carcinogenic activity, or to increase in quantity any such elements which might be in competitive activity with factors of a "virus-like" nature. It has been felt advisable to include a brief consideration of the theoretical approach to this problem as stated above. The following material is therefore divided into theoretical considerations, experimental approach and summary and conclusions.

THEORETICAL CONSIDERATIONS

According to Bayne-Jones (1937), malignancy may be defined as "a universal cell potentiality, varying in degree with cell type, of the nature of a somatic mutation, and resulting in autonomic proliferation independent of a continuously acting provocative agent." Although many doubtlessly have objected to the inclusion of the term "somatic mutation" in the above statement, a good, generally acceptable definition of malignancy is a necessary starting point for a consideration of just what malignancy implies, and just what are the causes, theoretical implications and practical approaches to the cancer problem.

On examination, a multiplicity of causes has resulted

in a multiplicity of theoretical interpretations and conclusions. As such varied interpretations have resulted in a vast number of experimental approaches, the following discussion is apologetically included.

At the present time, malignancy is considered by some to be the result of a primarily growth-inhibiting influence of carcinogens against which some cells, by means of a kind of "mutation," have developed a resistance to the growth-inhibiting effect, and have continued to proliferate in an uncontrolled manner. Others attribute all forms of malignancy to "viruses." Some, like Potter (1943), suggest the possibility of carcinogenic agents modifying normal cellular elements into "virus-like" substances which "compete with normal elements for building blocks." It should be included that there is also the possibility of carcinogenic activity modifying normal cellular elements in such a manner that critical controlling or growth-inhibiting mechanisms are inactivated, depleted or, somewhat similar to the first hypothesis, made ineffective. There are, of course, many other theoretical interpretations.

Proponents of the carcinogenic inhibition-escape interpretation of Haddon (1937, 1938) have considered the malignant cell to be modified in basic metabolic activity. Many propose to approach a solution of the cancer problem by subjecting such cells to materials which will interrupt such new uncontrolled metabolic activity.

Although it is questionable just what supporters of the "virus theory" have considered in regard to approaches to a solution of the problem, several things might be considered. If a virus is interpreted to be a complex physiological mass, depending on the utilization of specific cellular elements for its own proliferation, one is tempted to consider the possibility of a competitive removal of critical cellular elements as a disruption of a rather complex mass law reaction in which the removal of a right-hand product has stimulated left to right activity. This might lead to concurring increases of related

right-hand products which possibly contribute to increased cell size, and subsequent mitotic division to maintain adequate surface-volume relations, etc. An oversimplified interpretation of such a situation might be demonstrated as follows:

NORMAL: $A + B \rightleftharpoons C + D$

ABNORMAL: $A + B \longrightarrow C + DX$ Inactivation of "D" by competitive removal or modification:

Metabolites in constant demand. Cellular constituents constantly increasing in quantity—stimulating other processes to overactivity.

Whether such an interpretation may or may not be justified, approaches to the problem may here still lead to an attempted removal of the "virus" by destruction or modification by extrinsic agents, or, on the other hand, successful competition by the cell for common constituents by a bolstering of the quantity of normal competing agents by an extrinsic addition—leaving removal of the virus proper to cellular dynamics and normal destructive processes. If it is to be momentarily assumed that a possible modification, inactivation or depletion of normal controlling or growth-inhibiting mechanisms is within the limits of probability as a cause of uncontrolled proliferation (such a theoretical modification also possibly contributing to malignancy by its own proliferation or disruption of a complex mass-law equilibrium), the extrinsic addition of normal cellular elements is again to be considered.

The utilization of an extrinsic material to disrupt malignant processes and either selectively destroy such cells or convert them to the non-malignant state would appear to call for a consideration of whether one such extrinsic substance would ever suffice to adequately perform for all types of malignant cells and known virus-type tumors, or whether specific substances would be required for various cell types and viruses. This, of course, raises the question of whether malignancy is an

expression of one basic cellular disturbance manifestly brought about by various methods and agents. Experimental work with toxins and foreign materials also brings up the question of the actual differences which are to be found in the critical functioning processes of normal and malignant cells, and whether a material can be found which will ever selectively destroy the tumor cell and not the normal cell as well. Although reports occasionally give hope that promising results may some day come from this field of endeavor, it should be remembered that Haddon's interpretation of malignancy phenomena in itself has been repudiated by such workers as Morelli and Dansi (1939), Hearne-Creech (1936, 1939, 1940), etc.

With these many unanswered questions and problems, it might be well to direct attention to a consideration of the utilization of normal cellular components, and those instances where the extrinsic addition of cellular constituents would perform functions similar to those desired by foreign materials.

If the inability of normal controlling or growth-inhibiting mechanisms to function adequately may be taken as a basic consideration of the malignancy problem, there are three situations which should be appraised. If controlling mechanisms do not function, it is either because:

(1) Controlling mechanisms themselves have been basically modified in functional capacity or removed by direct or indirect physical-chemical activity of carcinogenic agents;

(2) Controlling mechanisms have become secondarily inactivated by either a functional or supply failure of materials which are utilized in conjunction with such controlling mechanisms; or

(3) Controls have become ineffective by the presence of a new abnormal stimulus which, for example, may take the form of a competitive disruption of the possible complex mass-law relationships mentioned before, and may be initiated by a competitive virus, or non-functional, proliferative elements which originated by direct or indirect carcinogenic activity. (It should, of course, be remembered that complex equilibrium relationships in themselves may constitute growth-controlling mechanisms in lieu of any specific controlling or inhibiting elements.)

In such conditions, the theoretical utilization of cellular components may be somewhat clarified by a review of the

following conclusions of Beadle and Tatum ("Genetic Control of Biochemical Reactions in Neurospora"):

(1) Expression of a given cellular character is usually the result of a number of biochemical reactions taking place in series or in parallel;

(2) Each reaction in the series is normally controlled by a specific gene, probably acting through a specific enzyme;

(3) If any step is blocked, the substance ordinarily acted upon in that step may accumulate; and

(4) If the immediate product of any single blocked step can be supplied artificially and utilized, the subsequent reactions proceed normally and the character expresses itself as though the step were not blocked.

If controlling mechanisms themselves have been inactivated or secondarily "blocked," the functional value of artificially supplying needed elements is apparent. What is more interesting to note, however, is that in the case of competitive agents, artificially supplied components not only would compete with such agents (whether they be true virus or modified cellular elements), but would supply blocked products and permit stabilization of any equilibrium disturbances which might have been responsible for uncontrolled proliferation.

It is to be remembered that causes of malignancy such as radiation and burning apparently act by destruction or modification of normal cellular elements. Fieser (1941) points out that carcinogenic hydrocarbons are characterized by their remarkable susceptibility to substitution reactions and surpass all other known aromatic hydrocarbons in this special form of chemical activity. They also enter into addition reactions rather sluggishly, and are attacked at points other than reaction centers indicated by substitutes. It is also interesting to note that under controlled laboratory conditions, specific amounts of purified hydrocarbons take rather definite periods of time to produce malignancy in specific species.

The transition of precancerous lesions to a final manifestation of malignancy raises many questions as to just what is happening in the cell:

(1) Is there an actual proliferation of any carcinogenetically affected elements?

(2) Is there a competitive replacement of normal elements by substances which might have been modified by carcinogenic activity?

(3) Could such a "competitive replacement" be a result of the non-utilization of elements because of an actual physiological modification resulting in a functional block with subsequent accumulation in relation to remaining normal elements (which gradually became inadequate in numerical and functional capacity)?

(4) Is the time element related to the speed at which such possible elements proliferate (being very slow, for example, in the case of an x-ray burn)?

(5) Could the time factor also be a corollary of the cells' ability to genetically provide new functional elements before becoming exhausted and/or overcome numerically or functionally by such modified components?

Just what is the genetic role of chromosomes and genes and just what relationship do they have with cellular functions:

(1) Does the genetic control of biochemical activity lie in the actual supply of necessary enzymes and related materials?

(2) Could the action of many specific carcinogenic agents actually be directed (directly or indirectly) against the ability or genetic mechanisms to provide such materials?

(3) Is the specific ability of a cell to provide definite quantities of special materials under certain conditions responsible for apparent genetic susceptibility to malignancy and related "lethal-gene" phenomena, etc?

Since central chromosomal factors are apparently embodied with the ability to reproduce themselves (as evidenced by normal mitotic processes), would not the capacity to meet cellular requirements be determined by both the original quantity of materials and their rate of proliferation—with a greater than normal demand possibly actually lowering the basic number of such central units? Gardiner (1937) observed that male mice receiving small amounts of estrogen possess mammary glands resembling those of normal virgin females and develop very few mammary tumors, while those receiving larger doses show abnormal growth and develop many tumors. Could an over-demand tax central genetic units to a point of functional depletion—and malignancy? Could such a theoretical explanation be applied to the development of mammary cancer by the aged human female?

As many normal cytoplasmic elements possibly also possess the ability to proliferate (as suggested by (1) the

work of Spiegelman (1945); (2) the above-mentioned theoretical indications that possibly carcinogenically modified elements also possess this ability; (3) the possibility that many cytoplasmic elements are merely proliferated duplications of central genetic units, etc.), would a period of low "demand" allow such cytoplasmic elements to maintain themselves, and, even in cases where actual proliferation might not exist, permit central genetic units to replenish themselves, build up a reserve, or, in the possibility of a new environmentally induced cytoplasmic function, work towards a gradual adoption of central genetic ability to carry on such new functional achievements?

It is interesting to note that Cowdry and Suntzeff (1944) showed that young New Buffalo mice (which were still being subjected to normal growth stimuli) developed a larger number of methylcholanthrene-produced epidermoid tumors than older mice (which theoretically might have had an opportunity to replenish the quantity of any critical malignancy-related materials). Although the explanation of the above observations might be more properly related to differences in degree of cellular activity than the above suggested interpretation, such dynamic processes conceivably account for actual increases and decreases in functional capacities, and, in view of the important role that environmental factors seem to play, constitute a normal, ever-active type of physical-chemical "somatic mutation" dependent on these environmental factors. In view of selective specificity, and the constant turnover of cellular components through cellular dynamics and the "dynamic state of equilibrium," it is also conceivable that resulting cellular-body fluid relationships exist which could minutely influence corresponding specific substances in related cell types, and offer an avenue by which such substances might also be gradually increased or decreased in quantity. If normal germ cells might be considered to be under such primary or secondary body-fluid influences, accumulative increases or

decreases in specific genetic elements might illustrate an example of hereditarily transmitted characters which originate from a constant, biochemical-mutative type of evolutionary development.

Such hypotheses offer theoretical explanations for many questions and problems: lethal gene phenomena, gray hair, bald heads—even the development and hereditary transmission of such diseases as diabetes. A complete list would be long, and, of course, would include the phenomena of old age. A satisfactory approach to malignancy is but one reason for desiring an answer to the question of whether cellular components actually can be artificially supplied and utilized!

Experimental Approach

As was just stated, primary objectives are related to the question of whether cellular elements can be artificially supplied to cells, and whether such elements can be functionally utilized. Experimental approaches involve many questions and problems—the necessity of supplying genetically specific substances which will not only be capable of performing desired functions, but will not be destroyed by the host cell; the preparation of a cell-free extract by processes which will not destroy desired elements, etc. The question even arises as to whether specific materials will remain functionally active in crude cellular extracts. The actual selective passage of sufficient elements through cellular membranes constitutes one of the biggest questions.

In turning to known malignancy phenomena, it is noted that upon exposure to cell-free extracts of similar virus-type tumor cells, normal cells selectively take up active virus. Although the validity of McIntosh (1933) and Carrel's (1925*a* and 1925*b*) work in similarly transmitting tumors induced by chemical carcinogenic agents is questioned, selective attraction and passage of specific materials is not only theoretically possible, but may be illustrated by many known physical-chemical expressions

of cellular dynamics. The exposure of malignant cells to normal cellular elements represents but another example—the situation merely being reversed in comparison to normal cells and malignant extracts.

Specific obstacles are probably not related to the size of specific particles as much as to an actual breaking up of critical physiological complexes. Other variables are undoubtedly related to quantities and comparative rates of proliferation, the latter being of special importance in regard to the capacity of specific materials to regain both the quantitative and subsequent functional dominance desired. Theoretically, it would be desirable to reintroduce enough normal elements to revert the cell to a benign, or at least precancerous state.

Circumstances made it necessary to limit present investigation to a simple preliminary experiment. Crude extracts were not supplemented by qualitative fractions. Desired tissue culture conditions, or even the use of anterior ocular chambers, had to be substituted by subcutaneous transplants of tumor cells.

Exact procedures involved the use of sterile trocars in subcutaneously transplanting small pieces of methylcolanthrene-induced squamous cell carcinoma of the Swiss mouse into normal five- to six-week-old mice of similar strain. Extracts were prepared by mechanically scraping normal squamous cells at room temperature from the shaven skin of normal mice, and mechanically grinding them at a temperature of from zero to four degrees Centigrade. Desirable intravenous injection of extract was not attempted because of physical and numerical limitations. Subcutaneous injection in and around transplants was discarded because of the number of experimental variables, and, as a result, the room temperature exposure of small tumor particles to extracts before transplantation was attempted. Advantages of such a procedure would be related to: (1) The ability to expose malignant cells to a high concentration of normal constituents; (2)

the convenience of differentiating the role of crude extracts with and without specific elements, etc.

Disadvantages are, of course, many: (1) The necessarily limited time of exposure; (2) possible decreased cellular metabolism and related activity; (3) the extremely small pieces of tumor required; (4) the increased opportunity for contamination to play a decisive experimental role, etc.

With a five- to six-hour exposure of pin-head size tumor particles, it was found that of a total of 110 tumors transplanted, approximately 25 per cent. of the 38 exposed to normal cellular extract, 80 per cent. of the 34 exposed to a malignant extract, and 70 per cent. of 38 exposed to a solution of physiological saline grew into normal-sized, palpable tumor masses by the end of a 2 to 3 week period.

One is justifiably hesitant in interpreting such results. Although very suggestive of theoretical phenomena indicated above, the necessarily limited number of test animals used is entirely too small for even attempting such an experiment, much less making definite conclusions. The same could be said for the inexperience of the operator, and, of course, the large number of variables and possible complications. In spite of this, it is felt that theoretical interpretations are sound and justify investigation of such phenomena and their application to cancer and related problems. If nothing more, it is hoped that the above efforts may serve as a stimulus for full-time workers and research teams to direct some of their energies towards further investigation of this subject.

Summary and Conclusions

An attempt has been made to analyze malignancy phenomena. Examination is made of: (1) Contemporary interpretations of fundamental causes and implications, (2) approaches to meeting the cancer problem in view of such interpretations, and (3) the role that normal cellular elements might play in practical therapeutic approaches.

Suggestions are forwarded in regard to the possible

role of genetics, comparative proliferation, "competitive replacement," etc. Theoretical hypotheses are suggested in explanation of related phenomena such as progressive, physical-chemical "mutation" and evolutionary development. The association of lethal-gene phenomena, geriatrics problems, the "anticipation" phenomena of such diseases as diabetes, etc., is indicated.

A preliminary test situation is described. Suggestive results are of value only as a stimulus for further investigations of this subject.

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THE MITOTIC INDEX IN THE CHICK EMBRYO

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By mitotic index is meant now[1] the per cent. of mitotic nuclei of any total number counted. The index is being used extensively to-day as a measure in studies on periodicity, mitotic stimulating activities of various drugs and hormones, etc., in many different organisms without due regard, in the opinion of the writer, for certain factors which affect the validity of the index. It seems important, therefore, to examine the nature of these factors in an effort to determine how valid the index may be in any case. We have, perhaps, more data which relate directly to this question in the case of the chick embryo than in any other organism. For this reason the following analysis has been restricted to such data.

Studies of the index in the chick embryo have led to definite conclusions about a number of important problems. Schultz (1918 and 1922) showed that there is a gradual decrease in the index between 18 and 72 hours of incubation and attributed this decline to a slowing down of the division rate. Stough (1935) concluded that the index is too low to account for known increase in nuclear number and in this conclusion found support for his hypothesis of modified mitosis. Derrick (1937) used the index in an analysis of early embryonic development.

Mitotic division of its nucleus is one phase in the life of a cell. The duration of this phase, that is, the time actually occupied by the process of mitosis, may or may not be related in a fixed way to the rest of cell time. The validity of the index as a measure of proliferative rate, however, depends upon an assumption. The truth of this assumption seems, tacitly, at least, to have been taken for granted by nearly all those who have used the index as such a measure. Coghill (1924) and Richards (1935), however, have pointed out that if both interkinetic time

[1] Minot (1908) used the expression "mitotic index" to denote the proportion of mitotic nuclei found among a thousand counted.

and duration of mitosis are prolonged or shortened proportionately the mitotic index is not changed.[2]

Do these quantities vary proportionately, or does either of them tend to remain constant? This question has been answered in the case of experimentally changed environmental conditions. Bucciante (1929) has shown that when chick eggs are incubated at temperatures slightly different from the optimum, development is slower or faster, as the case may be, but the mitotic index remains practically the same, *i.e.*, mitosis and interkinesis are affected equally, being prolonged or shortened proportionately. Such an experiment does not tell us, however, whether under normal conditions cells which undergo mitosis rapidly have shorter interkinetic periods than cells which have a longer duration.

The relationship between mitotic index, duration and the length of the cell cycle may be made clearer from the following considerations: If T represents the average time taken per cell to complete the full cycle from, say, telophase to telophase, t represents elapsed time and N the number of cells, then the number of cells will increase, if the divisions are distributed at random, as

$$N_t = N_o 2^{\frac{t}{T}}$$

If the duration of mitosis is D, all cells which were undergoing mitosis at time t will have divided at time $t + D$. The number of dividing cells is, therefore, equal to the difference between the cell counts at time t and $t + D$, or

$$N_{t+D} - N_t = N_o 2^{\frac{t+D}{T}} = N_t (2^{\frac{D}{T}} - 1)$$

and the mitotic index is represented by the equation

$$\frac{\text{mitotic index}}{100} = 2^{\frac{D}{T}} - 1 = e^{\frac{D}{T} \log 2} - 1 = .693 \frac{D}{T}$$

[2] A diurnal periodicity, if marked, would affect the reliability of the index as a measure of proliferative rate. The work of Steffins (1930) appears to indicate that no such periodicity exists in the chick embryo, so that this possibility may be dismissed.

Of the quantities involved in the above equation the one which has thus far been measured in the chick embryo most thoroughly is the mitotic index. The results to date are not promising, in so far as we might hope to use the index as a kind of biological constant. Different indices have been found at different ages and in different tissues, but, unfortunately, the indices may differ in counts made by different observers on the same tissue in embryos of the same age. For instance, in the case of the neural tube of the 48-hour chick Schultz (1918) found an average index of 6.8, and Woodard and Estes (1944) an index of 9.3. In the neural ectoderm of 33-hour chicks Schultz found an index of 4.5 and Stoddard (1929) of 2.4. It is possible, of course, that differences in results are due to variations in individual embryos, errors or variations in technic. A more probable explanation of such differences in the index will be mentioned later.

With respect to the duration of mitosis in the chick embryo the data available again show much diversity. Two types of method have been used to determine the duration—the direct and the indirect. The direct method involves simply observation and timing of divisions or phases of division in tissue culture. In using the indirect method the assumption is made that the fraction of the total time of mitosis spent by a given nucleus in a certain phase is equal to the ratio of the number of nuclei found in that phase to the total number of mitotic nuclei. If the time actually required for completion of one phase can be found, *e.g.*, from tissue culture observation, the total time for all phases can then be calculated.

Using the direct method, Levi (1916) found that mesenchyme cells of 3 to 17-day old chicks required a variable length of time to complete division, from 16 to 40 minutes, mostly 18 to 20. Strangeways (1923) observed that choroid cells required from 23 to 65 minutes, the average being 34 minutes. Fischer (1925) found 12 minutes. Olivo and Slavich (1930) found that two-day chick heart cells required 38 minutes to pass from the phase in which

the nuclear membrane disappears to that at which it is reformed. And, by the indirect method, W. and M. Lewis (1917) found that division of mesenchyme cells requires from 70 to 180 minutes. Wright (1925) found that in fibroblasts mitosis requires about 34 minutes. Stough (1935), using data from Stoddard (1929) calculated that the duration in neural tube cells is approximately 55 minutes.

The generation time has been estimated by several investigators. Strangeways (1923) found that cells in tissue culture divided once in 11 or 12 hours. Fischer (1925) reported division about once in 10 hours. These estimates agree very well, but Olivo and Slavich (1930) calculated that the average intermitotic time in chick heart cells *in vitro* is 20 hours on the second day and that it increases to 15 days by the time of hatching. In tissue cultures of 7-day chick myocardial cells, however, Olivo and Delorenzi (1932) observed a variation in the interkinetic time of from 7 to 21 hours.

The average length of the generation time may be calculated, if the number of cells present at two different times is known. Thus, if N_1 = the number of cells (mitotic and resting) present a time t and N_2 the number present at t_2, then

$$\frac{N_2}{N_1} = 2^{\frac{t_2 - t_1}{T}}$$

The data necessary for calculating T for the chick embryo are already available from the work of Schultz (1918). These data were published originally in sources not readily available. For this reason, that part of the data pertinent to the following discussion is here summarized in tabular form. To the original data of Schultz there have been added in this table certain calculations of my own.

Referring to the calculated values of T in this table, one notes the uniformity of the values for each of the time intervals covered by the data of Schultz. Since

these values are derived independently of either the mitotic index or of the duration and depend entirely upon fairly accurately measurable quantities, namely, cell number and time, it would appear that their close agreement must have some significance. In another count, which is not included in the table, Schultz found a total of 66,123 nuclei present in the nervous ectoderm of the 33-hour chick and a total of 196,772 in that of the 48-hour chick. These figures enable one to calculate the generative time and the duration for nervous ectodermal cells specifically, which are 9.5 hours and 44.7 minutes, respectively.

TABLE 1

SHOWING INCREASE IN CELL NUMBER OF CHICK EMBRYOS AT DIFFERENT AGES, TAKEN FROM SCHULTZ (1918), MODIFIED BY CALCULATION OF GENERATION TIME (T) AND TOTAL NUMBER OF CELLS FORMED FROM INITIAL NUMBER ACCORDING TO LOGARITHMIC GROWTH LAW

Age of embryo (hrs.)	Total number of cells	Mitotic index	T (hrs.) (cal.)	Total number of cells (cal.)
18–20	192,605	4.1	...	.....
33	405,814	2.1	13.0	428,600
48	907,413	3.1	13.1	969,500
72	3,349,703	1.7	12.9	3,532,000

Note, incidentally, in the table the rather remarkable agreement between the figures giving the total number of cells calculated for each of the three time intervals and the total number of cells actually counted by Schultz. The number calculated for 72 hours differs from the actual number counted by less than three per cent. This agreement indicates that the law of logarithmic growth used in calculating the number of cells in each case gives a truer picture of increase in cell number under the circumstances than the simple law of compound interest, used by Stough.

The value of T could be estimated in another and different way, if all cell division could be stopped at some phase which would be visible. The time required to produce such a complete block would be a direct measure of T. Woodard and Estes (1944) accomplished this in effect by

the use of colchicine. After 40 minutes' treatment the index rose from 9.3 per cent. to 13.6 per cent. Using this information in an extrapolation we get for T a value of 10.6 hours, which is in fair agreement with that observed or calculated in other ways.

This agreement on the length of the generative time, reached by three entirely different methods, suggests that the average length of the generative time for chick embryonic tissues of the age range involved is (except, perhaps, for myocardial cells) fairly constant. If this be true, it follows that a low mitotic index in a given case would correspond to rapidity of mitosis, *i.e.*, a short duration, and *vice versa*. The index would then not be a valid measure of proliferative rate or division activity upon the part of the cells concerned but only of the speed of the mitotic process, and then inversely.

Furthermore, if the length of the generative time is, on the average, within the order of magnitude here suggested, the calculations of Stough (1935) lose all value in so far as they purport to show that the mitotic division rate is inadequate to account for known increase in cell number. Let us take a single example from the calculations of Stough. Using the data of Schultz and the compound interest formula he calculated that in the period from 18 to 72 hours with an index of 2.7 there would be produced by 72 hours only 828,100 cells from the 192,605 counted by Schultz at 18 to 20 hours, instead of 3,349,703 actually counted by Schultz at 72 hours. Stough indicates, further, that, in order for this latter number of cells to be produced, either the mitotic index must be assumed to be 10.5, or, the index being actually 2.7, the duration must be as short as 30.8 minutes, a time which he considered impossibly short. From this and similar calculations Stough concluded that mitotic division is inadequate in amount to account for known increase in cell number. There must be then, according to Stough, some other kind of division in operation. This type of division, he concluded on these and other grounds, is a

kind of modified mitosis in which intranuclear division of the chromatin precedes the actual division of the nuclei (Stough, 1931).

There is here adduced, however, some indications that the generation time may be approximately fixed, and, if so, it follows that different mitotic indices would correspond inversely to different mitotic speeds. Indices as low as 2, and less, based on extensive counts in chick embryos, have been found. Calculation shows then that the duration of mitosis may be as little as 15 to 20 minutes in some cases. It is not impossible that some cells may complete division in as little as 30.8 minutes.

Finally, it may be pointed out that if the generation time has an average stability of the order suggested, variations in mitotic index and duration would be expected to be of comparable order. Calculation shows that all such values reported for chick tissues are so comparable, if the generation time is in the order of magnitude suggested. The lowest index noted in the literature (1.15, Schultz, 1918) and the highest (13.7, Derrick, 1937) correspond to mitotic durations of 10.2 and 136 minutes, respectively, if the generation time is taken to be 12 hours. These, in turn, agree fairly well with the reported extremes in duration: 12 minutes (Fischer, 1925), and 180 minutes (Lewis and Lewis, 1917).

In conclusion it may be pointed out that the foregoing analysis of the mitotic index is not intended to imply that the generation time is a fixed quantity but merely that within the early embryonic period it does tend to remain constant on the average. It may differ in different types of cells; it may change with age, as is suggested by the data of Olivo and Slavich already mentioned. This analysis does indicate, however, the way in which the index, the generation time and the duration of mitosis are related. It is obvious that the index cannot be used as a reliable comparative measure of proliferative rate without taking this relationship and these quantities into consideration.

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FURTHER STUDIES OF THE SALT MARSH SNAIL (*MELAMPUS BIDENTATUS* OR *LINEATUS*)

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IN 1932 the writer made an ecological study of the salt marsh snail (*Melampus bidentatus* or *lineatus*), the commonest form of Gastropod mollusc in the coastal salt marshes and the banks of tidal estuaries of the Atlantic seaboard. This present study deals with further work on the determination of the shell structure, the external anatomy of the animal, the anatomy of the head in particular, and some hibernation habits observed during the winter months.

Salt marsh snails are intermediate between marine and terrestrial forms, always found close to salt water and above the high tide line in situations not normally inundated at high tide but where the snails may be dashed with salt spray. The dominant plant growth in the marshes inhabited by *Melampus* is a coarse grass of the genus *Spartina*. Richards reports the unusual occurrence of *Melampus* on beds of mussels (*Mytilus edulis*) which were submerged between the tides. Although *Melampus* is regarded as a marine snail, it is a true air-breather unlike most of the forms closely allied to it, taking in oxygen from the air and giving off carbon dioxide through a true lung sac (Fig. 1) as in the true land snails.

The specific name *lineatus* is descriptive only of younger individuals and refers to the presence of darker transverse bands on the lighter shell-surface. Such bands vary in number from one to eight. In comparison with other Gastropod shells, those of *Melampus* are very thin and appear smooth, polished and banded when young. The younger ones show great variation in color, from almost black to darker and lighter shades of brown, and in others nearly creamy white. The usual color is a light

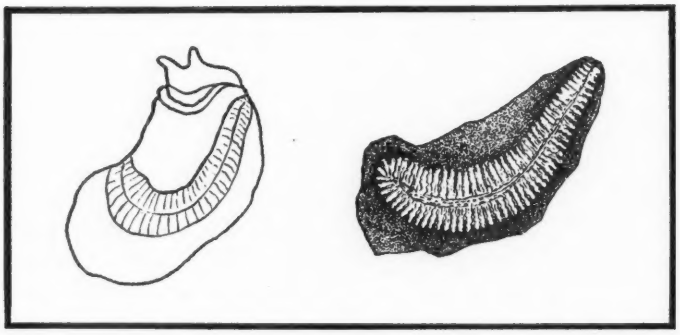

FIG. 1. Melampus removed from the shell showing the position of the lung sac. *Right:* The lung sac seen from the inner surface of the pigmented mantle.

yellowish brown with dark transverse bands. The shells of the adults possess no bands, and their surfaces are found, almost without exception, to be corroded and coated with muddy deposits. These characteristics of the adult shells tend not only to darken them but also to give them an apparent added thickness.

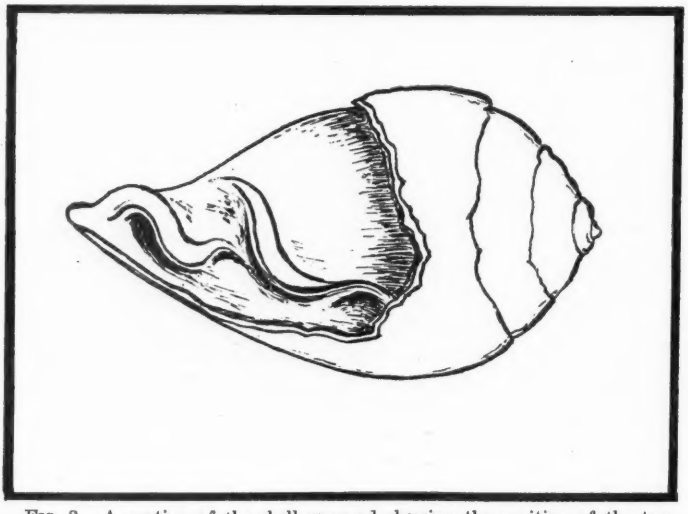

FIG. 2. A portion of the shell removed showing the position of the two teeth.

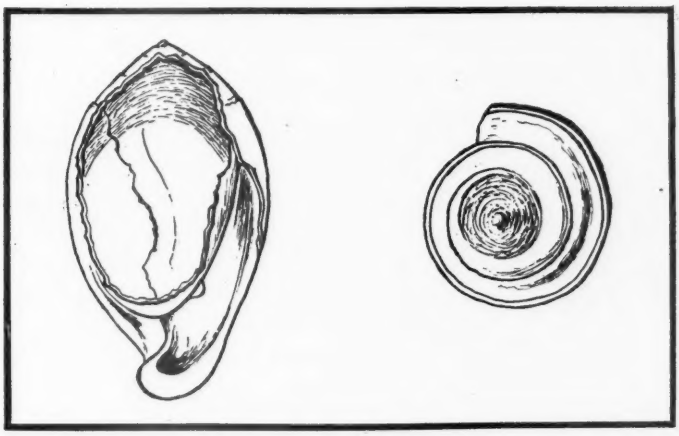

FIG. 3. Longitudinal section of the shell showing the absence of the internal partitions. *Right:* Cross-section of the shell looking into the spire.

Adult shells range in length from nine to twelve millimeters, possess a very short, compressed spire and a dextral slit-like elongate aperture bearing two white teeth on the left border (Fig. 2). The teeth probably serve as a barrier against predatory or intrusive insects. The

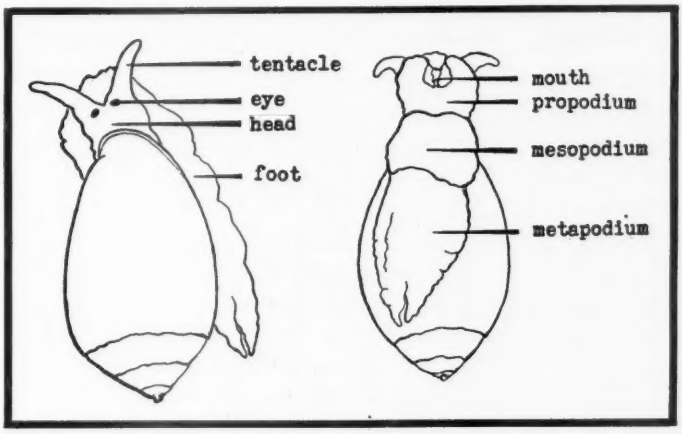

FIG. 4. Dorsal surface of fully extended individual. *Right:* Ventral surface of fully extended individual.

internal partitions of the spire of the shell are completely absorbed (Fig. 3), with the result that the body, at the apex, shows no spiral form and assumes the shape of the cavity in which it lies.

The head and foot when fully expanded and protruded from the shell reach a length of almost twenty-two millimeters. Dorsally the head bears two blunt tentacles with a black eye spot located at the base of each (Fig. 4). The foot, viewed from the ventral surface, is seen to be marked off into three well-defined regions (Fig. 4). These are

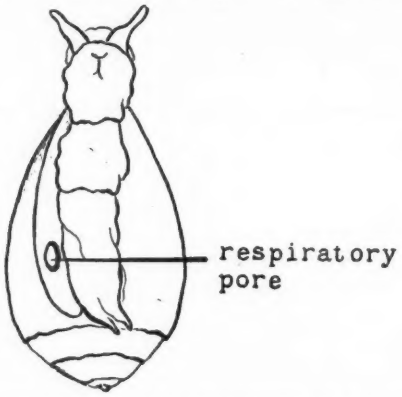

FIG. 5. Ventral surface showing the location of the respiratory pore.

called in other snails the propodium, the mesopodium and the metapodium. These terms are adopted by the writer for this species. The propodium is well developed, a condition reported to be true for representatives of those genera which crawl about in wet places, either muddy or sandy. The propodium is set off by a ventral transverse groove from the mesopodium and similarly separated from the metapodium. In locomotion, the animal uses the propodium as a sort of diminutive plow, pulling the rest of the body along after it, a motion suggestive of the looping action of some Lepidopteran larvae. The metapodium is divided at its posterior tip by a longitudinal groove. Hidden by the shell in dorsal view, and placed

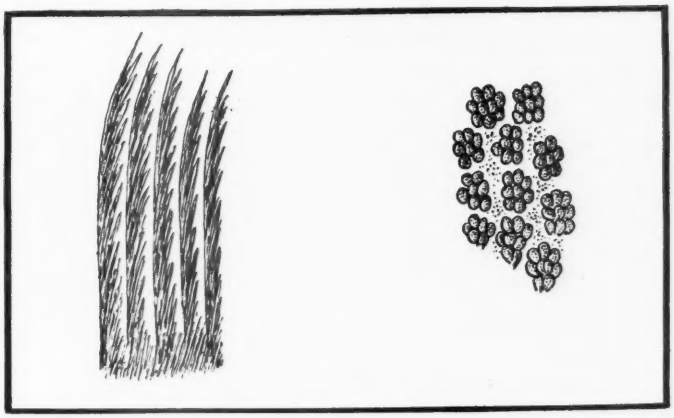

FIG. 6. The jaw.

to the right of the foot is a respiratory pore (Fig. 5) which opens and closes in rhythmic fashion.

The head bears on the ventral surface a mouth-opening surrounded by three thickened lips, one anterior and two lateral to the mouth. These lips, fringed with jaws (Fig. 6), open and close more or less rhythmically, scooping in the food as the animal moves slowly along. The radula (Fig. 7), very small, with tricuspid teeth set on a broad base, is located just within the mouth-opening at the entrance into the buccal mass, and forms the organ

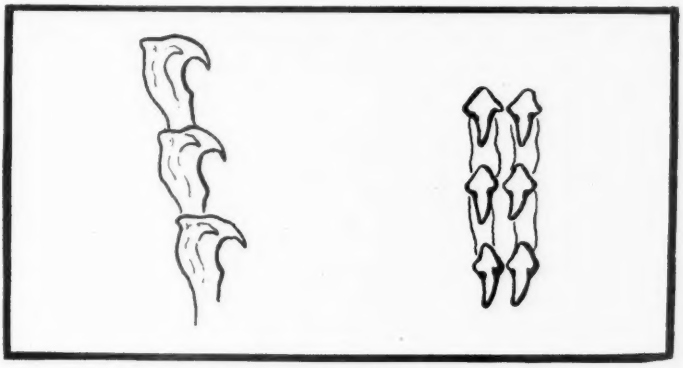

FIG. 7. The tricuspid teeth of the radula.

Table Showing Temperature Readings in Hibernation Areas of Melampus

Individual Hibernacula	Average temperature (from 5 readings) in degrees Farenheit
Base of matted and decaying Marsh Grass	31.4
Spongy roots of Marsh Grass	30.8
In cracks and crevices of mud (Depth of 1 - 2 inches)	30.5

Open-air temperatures at these localities, 1 foot above the grass, 20.0.

Average temperature on the surface of the mud 27.8, no snails found.

FIG. 8. Table showing temperature readings in hibernation areas of melampus.

for the rasping off and subsequent trituration of the food.

Records of the hibernation of *Melampus* were made in a coastal area near New Haven, Connecticut, during the month of December in the daylight hours when the average temperature was about twenty degrees Fahrenheit. The snails, in groups of a score or more, were found

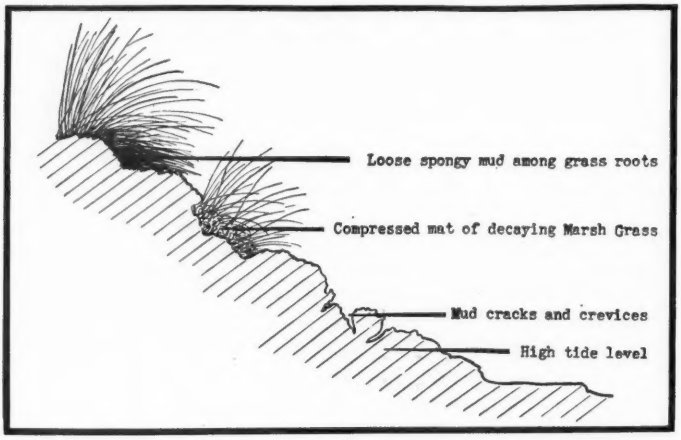

FIG. 9. Typical hibernacula of melampus in a salt marsh.

underneath thick tufts and mats of marsh grass (*Spartina*) especially where such compacted masses were decaying underneath, forming a compressed cushion of plant tissue. The oxidation produced in this organic mulch, as in silage, induced a surprisingly marked rise in the temperature to about 31.4 degrees Fahrenheit, which was 11.4 degrees over that of the surrounding air. Snails here were protected from the extreme cold and were provided with some food from the soft disintegrating plant tissues. Some snails were actively crawling about, and probably feeding, in the salt marsh silage. During

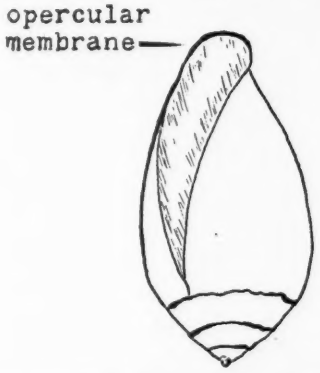

FIG. 10. Hibernating individual showing the opercular membrane closing the shell aperture.

very severe cold the animals were confined entirely to such areas, but on warmer days they emerged and crawled about feeding in the adjoining open muddy areas. Other hibernating places were in the loose spongy regions at the bases of the roots of the marsh grass, and also in crevices and holes in the mud located at depths of from one to two inches (Fig. 8). The temperature in these particular hibernacula were taken and compared with the open-air temperatures in the immediate region (Fig. 9).

In the inactive, hibernating individuals, the aperture of the shell was sealed over with a thin, transparent cellophane-like film known as the opercular membrane (Fig. 10), probably secreted by the foot, and differing

from the operculum in being a temporary secretion. This impervious membrane serves the double purpose of retaining a thin layer of warmer air immediately within the aperture of the shell, and of preventing the evaporation of moisture from the body.

The absence of an operculum in *Melampus* is an interesting feature since this structure is found in nearly all the land, the fresh-water and the marine snails. In these forms, the operculum affords the protection assumed, in part, by the teeth of *Melampus*. In those genera in which such a protective device is absent, the energies of the snails seem to be directed toward the thickening of the shell. In *Melampus,* however, this is not the case since the shell is notably thin, thickened only at the toothed aperture.

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